

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A01N 63/00, A61K 48/00, C07H 21/02, 21/04</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/33386</b> (43) International Publication Date: 6 August 1998 (06.08.98)
(21) International Application Number: <b>PCI/US98/01780</b> (22) International Filing Date: 30 January 1998 (30.01.98) (30) Priority Data: 60/036,321 31 January 1997 (31.01.97) <b>US</b> (71) Applicant (for all designated States except US): <b>VANDERBILT UNIVERSITY [US/US]; Suite 850, 101 21st Avenue South, Nashville, TN 37203 (US).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>BLASER, Martin, J. [-/US]; 733 Darden Place, Nashville, TN 37205 (US). THOMPSON, Stuart, A. [-/US]; 2423 Claylick Road, Whites Creek, TN 37189 (US). DWORKIN, Joel [-/US]; 718 Evans Avenue, Kirkwood, MO 63122 (US).</b> (74) Agent: <b>ADLER, Benjamin, A.; McGregor &amp; Adler, 8011 Candle Lane, Houston, TX 77071 (US).</b>			(81) Designated States: <b>AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</b>  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: <b>METHOD OF DELIVERING ANTIGENS FOR VACCINATION WITH A LIVE VECTOR</b>			
(57) Abstract <p>The present invention provides a mutant <i>C. fetus</i> strain in which each of the cassettes is replaced by a heterologous antigen. Also provided is a mutant <i>C. fetus</i> strain in which all but one of the cassettes are replaced by a heterologous antigen. Further provided is a mutant <i>C. fetus</i> strain in which <i>recA</i> is mutagenized and a <i>C. fetus</i> strain in which <i>recA</i> is mutagenized and the expressed <i>sapA</i> homolog is a chimera involving a heterologous peptide.</p>			

**BEST AVAILABLE COPY**

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

5

## METHOD OF DELIVERING ANTIGENS FOR VACCINATION WITH A LIVE VECTOR

10

### BACKGROUND OF THE INVENTION

#### Federal Funding Legend

15           This invention was supported in part under grant RO1-AI24145 from the National Institutes of Health. Accordingly, the United States government has certain rights in this invention.

#### Field of the Invention

20           The present invention relates generally to the fields of immunology and vaccine technology. More specifically, the present invention relates to methods of delivering antigens for vaccination with a live vector.

#### 25   Description of the Related Art

          One way in which microorganisms can alter their surface properties, allowing a fraction of the population to preadapt to environmental changes, is by varying protein expression through programmed genomic DNA rearrangements

30   (1). Phase, antigenic, or size variation of expressed surface proteins are governed by mechanisms including transposition and DNA inversion. During transposition, a silent gene is activated by movement to an expression site where it displaces the currently expressed gene. In DNA inversion, a segment of DNA is cut,

35   inverted, and then rejoined by a site-specific recombinase. The invertible DNA segment may contain either a promoter that directs expression of fixed structural genes or structural genes controlled by a fixed promoter. Transposition and inversion differ

in both the enzymes used and in the number of genes that can be controlled (many versus two).

*Campylobacter fetus*, a bacterial pathogen of ungulates and humans, is covered by a paracrystalline surface (S-) layer, composed of high molecular weight S-layer proteins (SLP) that masks most of the underlying gram-negative surface features (2). More than 300 bacterial genera have been described that possess S-layers (3). The S-layer renders *C. fetus* cells resistant to serum killing by prohibiting the binding of C3b (4), and the S-layer proteins themselves may change, permitting antigenic variation (5,6).

These S-layer proteins are encoded by 7-9 tightly clustered and partially homologous promoterless gene cassettes (7,8). Since previous studies show that *C. fetus* can express alternative S-layer proteins (4-6,9), there is only a single promoter for S-layer proteins expression present on a 6.2 kb invertible element (9), and the structural genes flanking the promoter are subject to substitution (9), both the promoter and the eight structural genes (*sapA* and its homologs) may rearrange strictly by inversion.

*Campylobacter fetus* is able to colonize the mucosa of the gastrointestinal and/or genitourinary tracts of mammals, birds and reptiles. Colonization of the wild-type organism lasts for years and can cause disease. Essential for this long term colonization is the ability to produce the S-layer proteins and *Campylobacter fetus* has the means to change the S-layer proteins and thus the crystal structure and the particular forms of antigenicity. This antigenic variation is required for the persistence of the organism in its environmental niche.

*Campylobacter fetus* accomplishes this antigenic variation by possessing 7-9 highly homologous gene cassettes, called *sapA* homologs (*sapA*, *sapA1*, *sapA2*, etc.) which encode a different S-layer protein. Each of these homologs contains a 5' region of about 600 base pairs which is completely conserved from homolog to homolog and is necessary for binding of the S-layer protein encoded by that homolog to the lipopolysaccharide molecule anchored in the bacterial outer membrane. The remainder of the open reading frame (ORF) is different for each

homolog but semi-conserved regions exist. Wild type *C. fetus* strains are able to rearrange their chromosomal DNA so that the *sapA* homolog positioned downstream of a unique promoter is then expressed. This rearrangement occurs at a frequency of  
5 about  $10^{-4}$  and is *recA* dependent. *RecA* is a protein encoded by *recA* and which is involved in homologous recombination and in repair of breaks of DNA strands.

*C. fetus* S-layer proteins (SLPs) are secreted in the absence of an N-terminal signal sequence. SLP proteins contain a  
10 signal sequence located within the C-terminous of the protein and are secreted through a type I protein secretion system encoded by the *sapCDEF* operon of four overlapping genes. Analysis of the C-termini of four *C. fetus* SLPs revealed conserved structures that are potential secretion signals. A *C. fetus sapD* mutant neither  
15 produced nor secreted SLPs. *E. coli* expressing *C. fetus sapA* and *sapCDEF* secreted SapA, indicating that the *sapCDEF* genes were sufficient for SLP secretion.

The prior art is deficient in the lack of effective means of delivering antigens for vaccination with a live vector. The  
20 present invention fulfills this longstanding need and desire in the art.

### SUMMARY OF THE INVENTION

25 In one embodiment of the present invention, there is provided a mutant *C. fetus* strain in which each of the cassettes is replaced by a heterologous antigen. The *sapA* homologs are altered by a DNA cassette inserted so that the encoded SLP represents a chimera between the native SLP and the peptide  
30 encoded by the cassette. The inserted DNA cassettes retain 3' *sapA* sequences that encode the C-terminal secretion signal sequences in order to ensure secretion of the chimeric protein. Representative examples of cassettes that can be inserted in this fashion include immunogens related to *Salmonella*, *Campylobacter*  
35 *jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) as well as other enteric, venereal, or respiratory pathogens of humans, cattle, sheep, poultry, horses, swine, and reptiles. In this embodiment of the

present invention, this strain can be used to immunize a host to develop mucosal and systemic immune responses to each of the immunogens.

5 In another embodiment of the present invention, there is provided a mutant *C. fetus* strain in which all but one of the cassettes are replaced by a heterologous antigen. In this embodiment, one of the cassettes (e.g., *sapA2*) remains in its native configuration and the others are mutagenized. The advantage of this construction is that it also can induce immunity  
10 to *C. fetus* based on the single full-length SLP produced.

In another embodiment of the present invention, there is provided a mutant *C. fetus* strain in which *recA* is mutagenized. When the RecA protein is not produced, the DNA rearrangements permitting *sapA* antigenic variation can not occur at any  
15 detectable frequency. Thus, the *C. fetus* strain can only produce one of the SLPs encoded by one *sapA* homolog. This strain can be used, therefore, to colonize the host briefly, i.e., until protective immunity has developed to that homolog. Subsequently, the host immune response eliminates the organism. Thus, this mutant  
20 would provide effective immunity against subsequent *C. fetus* infection and is useful for vaccination of ungulates (including sheep, cattle and horses) in which infectious abortion and/or infertility can occur after the wild-type infection.

In another embodiment of the present invention, there  
25 is provided a *C. fetus* strain in which *recA* is mutagenized and the expressed *sapA* homolog is a chimera involving a heterologous peptide. In this embodiment, a mutagenized *sapA* homolog expresses a chimeric protein including a heterologous antigen. The strain is then passaged *in vitro* so that  
30 the chimeric homolog is in the expression position and then a *recA* mutation is made. This strain now essentially expresses only the chimeric protein thus providing a means to immunize a host to that antigen. The duration of colonization in the host is brief and this attenuated *C. fetus* strain allows safe immunization  
35 for the selected antigen.

In yet another embodiment of the present invention, there is provided a mixed mutant *C. fetus* strains each including a *sapA* chimera which is also a *recA* mutant. In this embodiment,

mutants are constructed in which a single *sapA* homolog is mutagenized to encode a different chimeric protein representing a different heterologous antigen. Each mutant is also RecA-deficient due to mutation in *recA*. A host is inoculated with a mixture of two or more of these strains to provide immunization to the requisite antigens. Each strain is short-lived in the immunized host.

In another embodiment of the present invention there is provided a strain of *E. coli* carrying plasmids encoding the *sapCDEF* proteins that permits the secretion of chimeric proteins containing the SapA C-terminal secretion signal. The secreted protein is encoded by an altered *sapA* homolog that has been engineered so that the 5' section of the homolog-specific region is replaced by a DNA cassette encoding a heterologous antigen. Since the chimeric protein would be secreted by *E. coli* into the culture medium, this system provides a method for obtaining a large amount of the chimeric protein in a relatively pure form.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

### BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

**Figure 1** shows the schematic representation of serial experiments to utilize *km*- and *cm*-cassettes to examine S-layer proteins gene rearrangement (Figure 1A). Introduction of *cm* into strain 23D by marker rescue led to insertion into *sapA* to create

23D:AC100. This strain was selected on chloramphenicol-containing medium, and as expected was chloramphenicol (C) resistant, and was S<sup>-</sup> (no S layer) and not resistant to serum (S) and kanamycin (K), as previously reported (9). Incubation of  
5 23D:AC100 with normal human serum (NHS) selected for survivors (at a frequency of  $1 \times 10^{-4}$  (AC200 series) that were S<sup>+</sup> (S-layer present) and serum-resistant but sensitive to kanamycin and chloramphenicol. Strain 23D:AC200, expressing *sapA2*, was further mutagenized by introduction of *km* into *sapA2* to create  
10 the 23D:ACA2K100 series. These strains were S<sup>-</sup> and serum- and chloramphenicol- sensitive but kanamycin-resistant. Incubation with NHS selected for survivors (at a frequency of  $1 \times 10^{-4}$ ) that were S<sup>+</sup> and serum-resistant but kanamycin- and chloramphenicol-sensitive (ACA2K400 series). The reciprocal  
15 relationship between antibiotic- and serum-resistance suggests that only a single promoter for S-layer protein gene cassettes is present. All ACA2K400 series strains must have an S-layer protein gene cassette other than *sapA* or *sapA2* positioned downstream of the single S-layer protein promoter. The predicted  
20 genotypes are depicted. P, *sapA* promoter; bent arrow, location and direction of transcription of S-layer protein gene cassette; solid inverted triangles, antibiotic resistance gene insertion; NHS, normal human serum. Figure 1B shows the immunoblot of *C. fetus* strain 23D and selected mutants into which *cm* or *cm* and *km*  
25 were inserted into S-layer protein gene cassettes. As expected, all the serum-resistant strains expressed S-layer proteins (of 97 or 127 kDa) recognized by antiserum to conserved *C. fetus* S-layer proteins determinants whereas there was no expression for strains maintained on either antibiotic.

30 **Figure 2** shows the Southern hybridization of *HincII* (Figure 2A) or *PstI* (Figure 2B) digestions of chromosomal DNA from *C. fetus* 23D and ACA2K series mutants using probes to *km*, *cm*, the promoter region, the *sapA*-specific 3' region, the *sapA* 1-middle region, or the *sapA* 1-specific 3' region. Each probe  
35 hybridized to a single fragment regardless of the phenotype of the *C. fetus* strain. Figure 2C shows the mapping of S-layer protein gene cassette arrangement by PCR. PCRs were performed with template chromosomal DNA from strains 23D and ACA2K mutants



using *sapA*-specific 3' region forward (*sapA*) and *km* reverse (*km*) primers (left 4 lanes), *sapA1*-specific 3' region forward (*sapA1*) and *km* primers (middle 4 lanes), or *sapA* and *sapA1*-specific 3' region reverse (*sapA1*) primers (right 4 lanes). Figure 2D shows the cumulative restriction maps of the 4 strains presented in Figure 2A-2C. The location of the probes as indicated from the hybridizations is shown under the map for each strain. *sapA* x represents an uncharacterized S-layer protein gene cassette; arrows represent direction of transcription; solid lines represent expressed genes, dashed lines represent silent genes; P over bent arrow represents the *sapA* promoter; the heavy line represents the 6.2 kb invertible promoter-containing element, flanked by opposing S-layer protein gene cassettes. The asterisks represent the palindromic putative recombinase recognition sites (TTAAGGAaTCCTTAA) present in the 5' conserved region of each S-layer protein gene cassette (7), and restriction sites are indicated: H, *HincII*; N, *NdeI*; P, *PstI*.

Figure 3 shows the proposed model of molecular events involved in S-layer protein gene cassette rearrangement by DNA inversion. DNA inversion between two oppositely oriented cassettes follows DNA strand exchange at the putative recombinase target site (asterisk) found upstream of each S-layer protein gene cassette within the 5' conserved region (small grey box) (8). Patterned boxes represent variable regions of S-layer proteins gene cassettes. A 6.2kb intervening segment is topologically reversed leading to ordered rearrangement of the S-layer protein gene cassettes. Inversion of DNA segments containing the promoter ('P' over bent arrow) permits expression of alternate S-layer protein gene cassettes (mRNA, arrow). Illustrated are inversion of the 6.2 kb promoter-containing element alone (left), the 6.2 kb element and one (middle) or two (right) S-layer protein gene cassette ORFs and the resultant genotypes. Each of these genotypes has been observed (Figure 2D).

Figure 4 shows a schematic representation of the *sapA* invertible region, showing the *sapCDEF* genes, the locations of the divergent *sapA* and putative *sapCDEF* promoters (bent arrows), and the clones (pIR15, pIR13, pIR12, and pIR20) from

which the invertible region sequence was determined. The hatched areas represent the ca. 600 bp conserved regions at the 5' ends of sapA homologs flanking the invertible region (designated here as sapAx and sapAy), at which recombination occurs as the basis of sapA homolog rearrangements using S-layer protein antigenic variation.

**Figure 5** shows a phylogram generated by parsimony analysis, demonstrating the relatedness of ABC transport proteins from type 1 secretion systems. The percent of amino acid similarity (%Sim) and identity (%ID) with *C. fetus* SapD is shown at the right. The bold numbers adjacent to the phylogram branches indicate the percentage of 1000 bootstrap replicates supporting the clustering of those branches. Branches without bootstrap values were clustered in less than 50% of bootstrap replicates.

**Figure 6** shows an immunoblot detected with antiserum to SapA demonstrating the secretion of SapA from *E. coli* expressing *C. fetus* sapCEEf. One mg of whole cell proteins (lanes 1 and 2), or the amount of TCA-precipitate proteins present in 250 ml of culture supernatant (lanes 3 and 4) were analyzed. Lanes 1 and 3, C600 (pAMP1+pBCGYC1); and lanes 2 and 4, C600 (pIR100+pBCGYC1).

**Figure 7** shows several models of chimeric sap homologs for the use of *C. fetus* as a live vector for antigen delivery to the mucosal immune system. The left large gray box represents the N-terminal domain required for binding of the protein to the cell surface, the right sided smaller gray box represents the C-terminal domain required for secretion of the protein. The middle region shows the native sap homolog, and then in other examples, antigens related to influenza, HIV, Shigella, a model B-subunit of a toxin, or a model A subunit of a toxin. In the chimeras without the left sided gray box, the protein would be secreted without binding to the *C. fetus* cell surface. RecA at right refers to possible RecA status of the host *C. fetus* strain. If the strain is RecA<sup>-</sup> then it would only express a single cassette on its surface, but if RecA<sup>+</sup> (wild type), it would be able to switch the chimera it expressed.

## DETAILED DESCRIPTION OF THE INVENTION

The following abbreviations may be used herein: S-layer protein, surface layer protein; *km*, kanamycin-resistance gene; *cm*, chloramphenicol-resistance gene; NHS, normal human serum.

In the present invention, programmed gene rearrangements are employed by a variety of microorganisms, including viruses, prokaryotes, and simple eukaryotes, to control gene expression. In most instances in which organisms mediate host evasion by large families of homologous gene cassettes, the mechanism of variation is not thought to involve DNA inversion. *Campylobacter fetus*, a pathogenic gram-negative bacterium, reassorts a single promoter, controlling S-layer (surface) protein expression, and one or more complete open reading frames strictly by DNA inversion. Rearrangements are independent of the distance between sites of inversion. These rearrangements permit variation in protein expression from the large S-layer protein gene family and suggest an expanding paradigm of programmed DNA rearrangements among microorganisms. Furthermore, S-layer proteins were secreted from *C. fetus* via a type I protein secretion system encoded by the *sapCDEF* operon. Secretory signal sequences for the SapA family of secreted proteins was localized to the carboxy-terminus of these proteins.

The present invention is directed to a mutant *C. fetus* strain useful for vaccinating an animal to *Campylobacter fetus*, wherein said strain is mutated to contain a DNA cassette encoding a heterologous protein. In one embodiment of the mutant *C. fetus* strain, a *sapA* homolog is altered. In one embodiment of the mutant *C. fetus* strain, the heterologous protein is a S-layer protein. Preferably, the encoded S-layer protein represents a chimera between the native S-layer protein and the peptide encoded by the cassette. Preferably, the cassette is selected from the group consisting of *Salmonella*, *Shigella*, *Campylobacter jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and other animal pathogens. In one embodiment of the mutant *C. fetus* strain, the cassette contains a 5' binding region and 3'

secretion signal region and the protein is inserted between said binding region and said signal region. In another embodiment of the mutant *C. fetus* strain, the cassette contains a 3' secretion signal but has no binding region. Preferably, the protein is an antigen or a therapeutic agent.

The present invention is also directed to a method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of the strain described herein.

The present invention is also directed to a mutant *C. fetus* strain, wherein *recA* is mutated so that no functional RecA protein is produced, the DNA rearrangements permitting *sapA* antigenic variation occur at a very low frequency and wherein said *C. fetus* strain can only produce one of the S-layer proteins encoded by one *sapA* homolog. In one embodiment, this strain contains a *sapA* homolog expressing a chimeric protein including a heterologous antigen.

The present invention is also directed to a mixture of mutant *C. fetus* strains, wherein each strain includes a *sapA* chimera which is also a *recA* mutant, wherein a single *sapA* homolog is mutated to encode a different chimeric protein representing a different heterologous antigen and each mutant is also RecA-deficient due to mutation in *recA*. The present invention is also directed to a method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of these strains.

The present invention is also directed to a strain of bacteria modified to express *SapCDEF* genes. Preferably, the strain is *Escherichia coli*. In one embodiment of this strain, a heterologous protein is expressed as a chimeric protein composed of sequences of heterologous origin, sequences that direct the secretion of said chimeric protein to the cell surface and sequences that direct the binding of the secreted chimeric protein to the lipopolysaccharides of the bacterial cell surface via the *sapCDEF* directed type 1 secretory system. The present invention is also directed to a method of immunizing a host to generate immune responses to an

immunogen., comprising the step of administering to said host a pharmacologically effective dose of this strain.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not  
5 meant to limit the present invention in any fashion.

### EXAMPLE 1

#### Bacterial strains and culture conditions

Wild-type S<sup>+</sup> (possessing an S-layer) *C. fetus* strain  
10 23D and spontaneous S<sup>-</sup> (no S-layer present) mutant strain 23B have been extensively characterized (4,5,12,13). Other *C. fetus* strains used were defined mutants derived from strain 23D, as described below. Stock cultures were stored and grown as described elsewhere (8). PVNT media containing 7 U/ml  
15 polymyxin B, 10 µg/ml vancomycin, 50 µg/ml naladixic acid, 10 µg/ml trimethoprim lactate, were supplemented for kanamycin-resistant strains or chloramphenicol-resistant strains, with 30 µg/ml kanamycin or 15 µg/ml chloramphenicol, respectively. *E. coli* strains used, including DH5α, HB101 and XL1-Blue  
20 (Stratagene, La Jolla, Calif.), were grown in L broth or on L plates (14).

### EXAMPLE 2

#### Chemicals and enzymes

25 Isopropyl-β-D-thiogalactopyranoside (IPTG) (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) (28 µg/ml) were purchased from Jersey Lab Supply (Livingston, NJ.). Restriction enzymes, T4 DNA ligase, Taq polymerase and *E. coli* DNA polymerase large (Klenow) fragment were from Promega and  
30 U.S. Biochemical Corp. (Cleveland, OH.). Antibiotics were from Sigma Chemical Co. (St. Louis, MO.), and [α-<sup>32</sup>P]dATP (650 mCi/mmol) was from ICN Radiochemicals (Irvine, Calif.).

### EXAMPLE 3

#### Genetic techniques

Chromosomal DNA was prepared from 48 hour plate cultures, as described (9). Plasmids were isolated by the

procedure of Birnboim and Doly (15). All other standard molecular genetic techniques were done, as described (14).

#### EXAMPLE 4

##### 5 Construction of mutant *C. fetus* strains

Mutant *C. fetus* strains were created by mobilization of donor pKO500 or pKO505 plasmid constructs by conjugal mating as described elsewhere (9) with sequential selection (as shown in Figure 1) on media containing 30 µg/ml kanamycin or 15 µg/ml chloramphenicol, or in the presence of 10% normal human serum (NHS), as described (16). pKO500: suicide hybrid plasmid with the *sapA* open reading frame disrupted with a chloramphenicol-resistance gene (*cm*) (17) located 127 base pairs into the open reading frame. pKO505: suicide hybrid plasmid with the *sapA* 2  
10 open reading frame disrupted with a promoterless kanamycin-resistance gene (18) located 127 base pairs into the open reading frame .  
15

#### EXAMPLE 5

##### 20 Bactericidal assays

To determine the susceptibilities of the mutant strains to the bactericidal activity of normal human serum, 10-fold serially-diluted cultures (starting from a single colony) were incubated at 37°C for 60 minutes in the presence of 10% pooled  
25 normal human serum or 10% heat-inactivated normal human serum, as described (9, 16). Wild-type S<sup>+</sup> strain 23D and spontaneous S<sup>-</sup> mutant strain 23B were the serum-resistant and serum-sensitive controls, respectively (9). Cultures then were plated to media containing chloramphenicol, kanamycin, or no  
30 antibiotic selection (Figure 1A), and following incubation, bacterial colonies were enumerated. Survival rates were determined as the ratio of colony forming units (cfu)/ml in the presence of normal human serum or heat-inactivated human serum, or a similar ratio of cfu/ml in the presence or absence of the selective antibiotic.

35

#### EXAMPLE 6

##### Production of antiserum to *C. fetus* S-layer proteins

Antiserum to the 97 kDa S-layer proteins of type A strain 82-40LP was raised in adult New Zealand White female

rabbits and shows broad recognition of *C. fetus* S-layer proteins as described (5). To analyze wild-type and transconjugant *C. fetus* strains for S-layer proteins expression, cells were harvested from plates, lysed in sample loading buffer and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed as detailed (5,13).

### EXAMPLE 7

#### 10 Southern hybridizations and probes

*C. fetus* chromosomal DNA was digested with *HincII* and processed exactly as described (12). Probes included the gel-purified PCR products specific for the *sapA* promoter region (9), *km*, *cm*, 3' *sapA* region (1649 to 2760 bp), middle *sapA1* region (620 to 1381 bp) and 3' *sapA1*-region (1381 to 2763 bp). Probes were <sup>32</sup>P-radiolabeled by primer extension with random hexameric oligonucleotides (19). Polymerase chain reactions were performed as described (9).

### 20 EXAMPLE 8

#### Polymerase chain reaction

Polymerase chain reactions were performed as described (9). Amplification of large PCR products was accomplished by denaturing for only 5 seconds and extending at 68°C for longer periods, typically 4-7 minutes. Primers used include:

*km* F, 5'-TG TAGAAAAGAGGAAGGAAA-3' (SEQ ID No: 1);  
*km* R, 5'-CTAAA ACAATTCATCCAGTA-3' (SEQ ID No: 2);  
 30 *cm* F, 5'-AGTGGATAGATTTATGATATAGTG-3' (SEQ ID No: 3);  
*cm* R, 5'-T TTATTTATTTCAGCAAGTCTTG-3' (SEQ ID No: 4);  
*sapA* F middle, 5'-CATCTCTACAGCAGCAAAAG-3' (SEQ ID No: 5);  
*sapA* F 5'-GCGGAGATAATGTTGTAGTTGAT G-3' (SEQ ID No: 6);  
 35 *sapA* R, 5'-AACTTTAAGAT CTAGCGTACC-3' (SEQ ID No: 7);  
*sapA1* F middle, 5'-AGGGTACTGATTTAGACGATA-3' (SEQ ID No: 8);

*sapA1* F 3', 5'-GCTGGATTACAGGAGATTTAACC-3' (SEQ ID No: 9);

*sapA1* R 3' #1, 5'-GTTACTGGTATCAATAA CAACATAAGT-3' (SEQ ID No: 10);

5 *sapA1* R 3' #2, 5'-CTACGTAATCATACTGCTACC-3' (SEQ ID No: 11).

### EXAMPLE 9

#### 10 Construction and phenotypic analysis of mutant strains

To show that both the *sapA* promoter and the complete structural genes can be mobilized by DNA inversion, mutant strains were created in which both S-layer protein-encoding gene cassettes bracketing the invertible *sapA* promoter were disrupted (Figure 1A). First, insertion of a chloramphenicol-resistance cassette (*cm*) into the open reading frame (ORF) of the expressed S-layer gene cassette (*sapA*) ablated SapA expression (Figure 1B) and rendered the organism (23D:AC100) serum-sensitive. Using the ability of S<sup>+</sup> (but not S<sup>-</sup>) *C. fetus* strains to survive incubation in normal human serum (4, 13, 20-22), mutants (9) expressing the *sapA2* cassette were then selected for promoter inversion and identified strain 23D:AC200 (Figure 1). Next, 23D:AC200 was mutagenized by insertion of the kanamycin-resistance cassette (*km*) to ablate SapA2 expression, creating 25 23D:ACA2K101 (Figures 1 and 2D). Incubation of this dually disrupted strain in serum selected for serum-resistant survivors. These were found at a frequency of  $1 \times 10^{-4}$ , and as expected, expressed S-layer proteins of 97 kDa or 127 kDa (Figure 1B) and were antibiotic-sensitive. Immunoblot of 26 serum survivors 30 demonstrated that 16 (62%) expressed a 97 kDa (e.g., 23D:ACA2K400) and 10 (38%) expressed a 127 kDa (e.g., 23D:ACA2K450) S-layer proteins. These results suggested that in each of the survivors there had been exchange of at least one of the bracketing cassettes. None of the mutant strains maintained 35 on antibiotic-containing media produced an S-layer proteins band, as revealed by immunoblot, (Figure 1b), and this shows that expression of *cm* or *km* is dependent on the single *sapA* promoter. These experiments provided a group of well-defined strains with



which to examine the genotypic events associated with the observed phenotypic variations.

### EXAMPLE 10

5

#### Phenotypic variation is associated with nested DNA inversions

To investigate the nature of the recombination event that allowed the single *sapA* promoter to express a native S-layer gene cassette in the dually disrupted strains, using Southern analyses, these organisms were compared with wild-type *C. fetus* strain 23D and the S<sup>-</sup> parental mutant 23D:ACA2K101. The known positions of *HincII* (Figure 2A), *PstI* (Figure 2B), and *NdeI* (data not shown) sites located in *sapA*, *sapA1* and *sapA2* (8,9,23,24) were used to predict the size of the fragments hybridizing with appropriate probes. The promoter region, the *sapA*-3' region, the *sapA1* middle region and the *sapA1*-3' region probes hybridized to 8.5 kb, 4.8 kb, 4.8 kb and 4.3 kb *HincII* fragments, respectively, in wild-type strain 23D (Figure 2A). Based on these and previous Southern hybridization and PCR data (9), the location of these genes relative to one another in wild-type strain 23D was defined (Figure 2D, top line).

An identical hybridization pattern was observed for mutant 23D:ACA2K101 with the exception that the *km* and *cm* probes hybridized to a 10.4 kb fragment and that the promoter region probe also hybridized to a 10.4 kb fragment, (Figure 2D, line two). These results are entirely consistent with the introduction of the *km* (0.8 kb) and *cm* (1.1 kb) markers flanking the promoter. For the mutant strains ACA2K400 and ACA2K450, changes in phenotype were clearly associated with change in probe co-hybridization (Figure 2A). In mutant strain 23D:ACA2K400, the *km* probe hybridized to the same 5.1 kb fragment as the *sapA*-3' region probe and in strain 23D:ACA2K450 to the same 4.0 kb fragment as the *sapA1*-3' region probe (Figure 2A), whereas the promoter region probe hybridized to fragments larger than 9 kb. For each of the mutant strains, the *cm* and promoter region probes hybridized to fragments of identical sizes (Figure 2A). These results demonstrate that the open reading frames had inverted (Figure 2D), and reflect the differing locations

of each of the S-layer protein gene cassettes in relation to the *km* marker. The total size of non-overlapping hybridizing fragments remained constant among the strains (17.6 kb in wild-type and, reflecting addition of the two antibiotic resistance cassettes, 19.5  
5 kb in all mutant strains) indicating that recombination did not involve net duplication or deletion of DNA (Figure 2d).

Following *Pst*I digestion, as expected, the *km* probe hybridized to the same 7.3 kb, 6.5 kb, and 5.9 kb fragments as the promoter region probe, the *sapA*-3' region probe and the *sapA*1-3'  
10 region probe in strains ACA2K101, ACA2K400 and ACA2K450, respectively (Figure 2B). Despite the genotypic differences among the mutant strains, the constancy of the 3.1 kb fragment hybridizing with the *cm* probe (Figure 2B) was consistent with the position of the marker downstream of the promoterless end of the  
15 6.2 kb invertible element (Figure 2D). Hybridization of the promoter region probe to 5.5 kb, 4.9 kb and 5.3 kb fragments in strain 23D and mutants ACA2K400 and ACA2K450, respectively, also reaffirms the model. The *sapA*-3' region probe hybridized with a 4.1 kb fragment in strains 23D, and mutants ACA2K101  
20 and ACA2K450, but not ACA2K400 (Figure 2B), consistent with the locations of *Pst*I sites within *sapA* and *sapA*1, and the tandem relationship between these two cassettes. Similarly, the *sapA*1-3' region probe hybridized with a 4.2 kb fragment in strains 23D, ACA2K101 and ACA2K400, but not ACA2K450 (Figure 2B),  
25 consistent with the tandem relationship between *sapA*1 and the downstream *sapA*x in the first three strains. The observed sizes of each of the hybridizing fragments and their co-hybridization patterns in *Pst*I (Figure 2B) or *Nde*I-digested (not shown) genomic DNA using similar probes were completely consistent with the  
30 known restriction sites (8,9,23,24) as depicted in Figure 2D.

PCR analyses provided independent confirmation for open reading frame inversion. The 3' *sapA*-specific forward primer and a *km* reverse primer yielded a product for strain 23D:ACA2K400 (Figure 2C), indicating that *sapA* had inverted and  
35 was located immediately upstream of *km*. Similarly, for strain 23D:ACA2K450 the data (Figure 2C) indicate that *sapA*1 had inverted and was located immediately upstream of *km*. PCR analyses using the 3' *sapA*-specific forward primer and 3' *sapA*1-

specific reverse primer yielded a 4.3 kb product for all strains except for 23D:ACA2K400 (Figure 2C, rightmost 4 lanes), indicating that the tandem relationship of *sapA* and *sapA1* was lost in strain ACA2K400. The data indicate that for mutant strains  
5 23D:ACA2K400 and 23D:ACA2K450, *sapA*, or both *sapA* and *sapA1*, respectively, have inverted in relationship to *sapA 2* (Figure 2D, lines 2-4).

### EXAMPLE 11

10

#### DNA inversion events occur independent of the distance between recombination sites

The frequency of the DNA inversion events was determined, involving promoter alone, or promoter and one or  
15 more of the gene cassettes. The mutant strains (TABLE 1) provided easily definable phenotypes with which to assess the inversion events. These were measured by conducting experiments in which the strains were examined for resistance to a selection that would be lethal (exposure to kanamycin,  
20 chloramphenicol, or serum) unless defined inversions allowing expression of genes to overcome the exposure had occurred. Events involving inversion of the promoter-containing element alone, or together with one or two open reading frames, occurred at nearly equal frequency ( $\sim 1$  to  $2.6 \times 10^{-4}$ ) (TABLE 1). These  
25 data imply that inversions involving two adjacent open reading frames occurred in a single event and did not result from two or more sequential inversion events. Recombination occurs at either homologous or palindromic DNA sequences (9). The distance-independence between sites suggests that inversion occurs by a  
30 random collision model, as proposed (25).

TABLE 1

C. fetus survival after serum or antibiotic selection

5		Relevant Phenotype					
	<u>Strain</u>	<u>Susceptibility to</u>			<u>Immunoblot</u> presence of <u>S layer: kDa</u>	<u>Selection*</u>	No. <u>Expt</u>
		<u>Chlor.</u>	<u>Kana.</u>	<u>Serum</u>			
10	23D:AC100	R	S	S	---	Serum	7
	23D:AC200	S	S	R	127	Chloram.	6
	23D:ACA2K101	S	R	S	---	Chloram.	12
	23D:ACA2K101	S	R	S	---	Serum	8
	23D:ACA2K200	R	S	S	---	Kanamycin	3
15	23D:ACA2K400	S	S	R	97	Kanamycin	6
	23D:ACA2K400	S	S	R	97	Chloram.	6
	23D:ACA2K450	S	S	R	127	Kanamycin	5
	23D:ACA2K450	S	S	R	127	Chloram.	6

S: sensitive; R: resistant; Chloram.: chloramphenicol. \*Selection by plating cells

20 with 15 µg/ml chloramphenicol or 30 µg/ml kanamycin, or incubating cells in 10% NHS

## EXAMPLE 12

### Cloning of the invertible region

Since bacterial genes involved in similar functions are often clustered, the 6.2 kb invertible region between two *sapA* homologs was characterized. The 6.2 kb fragment was first amplified by PCR, and this product was subcloned into pAMP1 to yield the plasmid pIR100. Next, this subcloned fragment was used as a probe to isolate a series of overlapping plasmid clones derived from a *C. fetus* 23D genomic library constructed in  $\lambda$ ZAPII. Four of these, designated pIR15, pIR13, pIR12, and pIR20 represented the majority of the invertible region and were subjected to DNA sequence analysis. In order to determine the sequence of the small sequence gap between pIR13 and pIR12, a DNA segment was amplified from *C. fetus* 23D genomic DNA by PCR using appropriate primers and subcloned into pT7Blue. To avoid the problem of PCR-induced errors, three independent subclones were sequenced and in each case the sequence was identical.

## EXAMPLE 13

### Analysis of invertible region features

The DNA sequence of the 6229 bp invertible region from strain 23D was predicted to contain four open reading frames, which were designated *sapCDEF* (Figure 4). The *sapC* gene began 596 bp from the initiation codon of the oppositely-oriented upstream *sapA* homolog. The *sapC* open reading frame was 1035 bp in length and was immediately followed by the *sapD*, *sapE*, and *sapF* open reading frames, which were 1752, 1284, and 1302 bp, respectively. Each gene in this cluster had a typical ribosome binding site, and overlapped the preceding gene, by 14 bp (*sapC/D*), 1 bp (*sapD/E*), and 11 bp (*sapE/F*). The *sapF* gene ended 287 bp upstream of the *sapA* homolog located downstream. The 74 bp preceding the ATG codons initiating translation of the *sapA* homologs flanking *sapCDEF* were identical to each other (Figure 4). These conserved segments have previously been noted upstream of the three characterized *sapA* homologs, and may play a role in

the inversion of this DNA segment. As a potential component of this recombination mechanism, sequences resembling  $\chi$  sites (RecBCD recognition) were present at positions 31-38 and 6192-6199. Several potential  $\sigma^{70}$ -like promoters were noted 44-243 bp upstream of *sapC*. These putative *sapCDEF* promoters were oriented in the opposite direction to the *sapA* promoter, with the two -35 regions separated by 200-380 bp. Due to the overlapping nature of the *sapCDEF* genes and the lack of other putative promoters, it is likely that they are co-transcribed.

10

### EXAMPLE 14

#### Similarities of SapCDEF to other proteins

The *sapC* open reading frame predicted a protein product of 344 amino acids (39.7 kDa) that had no significant similarities in the non-redundant database maintained by NCBI. In contrast, the products of the *sapDEF* genes had high similarity to proteins encoding type I secretion systems. SapD (584 amino acids, 64.0 kDa) was related to the ABC family of transporters, especially those that are involved in the translocation by type I secretion systems of proteases, lipases, hemolysins, and leukotoxins across the envelopes of gram-negative bacterial pathogens. The degree of similarity between SapD and these proteins was between 68% similarity (with *Pseudomonas aeruginosa* AprD) and 50% similarity (*Actinobacillus actinomycetemcomitans* LktB). In addition, SapD contained two motifs found in such proteins, an ATP/GTP binding site (GPSAAGKS; Walker Box A, amino acid residues 365-377) and a peptide (LSGGQRQRVALA, amino acid residues 468-479) that is a signature sequence for ABC transporters. The SapE protein (428 aa, 47.9 kDa) was similar to the MFP proteins of type I transporters, typified by *P. aeruginosa* AprE (52% similar) and *E. coli* HlyD (49% similar). SapF (434 amino acids, 49.4 kDa) was related to the outer membrane component of type I systems, such as *P. aeruginosa* AprF (45% similar), *Erwinia chrysanthemi* PrtE (41% similar), and *E. coli* TolC (47% similar). Parsimony and bootstrap analyses supported the classification of the *C. fetus*

transport proteins on phylogenetic branches distinct from other type I secretion apparatuses (Figure 5).

### EXAMPLE 15

#### 5 Construction of a *sapD* mutant

To examine whether genes in the *C. fetus* invertible region encoded proteins responsible for SLP secretion, a derivative of type A strain 23D containing an insertional mutation in *sapD* was constructed. To generate a *sapD* mutation, an *aphA* cassette was inserted into a unique BglII site within *sapD* and transformants were selected on plates containing kanamycin. This new clone was designated pIR131. The *sapD* insert containing the resistance fragment was subcloned into suicide vector pILL570 to yield pILL131, which was transformed into *E. coli* S17-1. Transformants were selected from trimethoprim/kanamycin plates and verification of pILL131 was made by digestion with *HindIII*. The *mob*<sup>+</sup> *E. coli* strain S17-1 containing IncP DNA transfer functions and pILL131 was used as the conjugation donor, and *C. fetus* wild-type strain 23D was the recipient. Approximately five thousand transconjugants were recovered (frequency of  $4 \times 10^{-6}$  transconjugants per recipient). Chromosomal DNA from one of these strains was extracted and digested with *NdeI* for Southern analysis, using hybridization probes for *sapD*, *aphA*, and pILL570. The *sapD* probe hybridized with a 4.2 kb *NdeI* fragment in wild-type strain 23D, exactly as predicted from the DNA sequence of the invertible region. The size of the *NdeI* fragment in strain 97-205 hybridizing to the *sapD* probe was 5.6 kb indicating the incorporation of the 1.4 kb *aphA* cassette in *sapD*, as expected. The hybridization of the *aphA* probe with a fragment of 5.6 kb in 97-205 but not in 23D was consistent with this observation. A pILL570 probe did not hybridize with DNA from 97-205, indicating that 97-205 was derived from a double crossover event in which only the mutagenized *sapD* allele was incorporated into the chromosome. PCR experiments using *sapD*- and *aphA*-specific primers confirmed the Southern hybridization data. These results indicate that a strain containing an insertional mutation in *sapD* has been successfully constructed.

## EXAMPLE 16

### Properties of a *C. fetus* *sapD* mutant

Whether the *C. fetus sapD* mutant strain 97-205 was  
5 able to export S-layer proteins and to assemble a functional S-layer on its surface was next determined. First, the ability of the wild-type and *sapD* mutant strain to resist complement-mediated killing, a phenotype consistent with the presence of the S-layer was examined. Suspensions of cells were exposed either to normal  
10 human serum or to heated-inactivated normal human serum, and the extent of complement-mediated killing was determined. As expected, the S<sup>+</sup> strain 23D was completely resistant to killing (0.31 log<sub>10</sub> kill). In contrast, as expected, there was approximately three log<sub>10</sub> killing of strain 23B (3.34 log<sub>10</sub> kill), that is unable to  
15 express an S-layer. Results for strain 97-205, the *sapD* mutant (3.04 log<sub>10</sub> kill), were nearly identical to that for 23B, and are consistent with the absence of a functional S-layer on the surface of *sapD* mutant strain 97-205.

To understand the basis for the serum-susceptibility of  
20 the *sapD* mutant, immunoblots were performed to detect the presence of SLPs on the cell surface. S-layer proteins can readily be removed from the surface of S<sup>+</sup> cells by washes with distilled water. Therefore, water washes and whole-cell proteins of wild-type and *sapD* mutant cells were analyzed by immunoblot with  
25 polyclonal antiserum against SLPs. In the wild-type strain, S-layer proteins were detected both in whole-cell samples and water washes, as expected for cells expressing a functional S-layer, whereas no S-layer proteins were detected for S<sup>-</sup> strain 23B. In the *sapD* mutant, however, S-layer proteins were not detected  
30 either extracellularly or in whole-cell samples. Thus, disruption of *sapD* by the insertion of an antibiotic-resistance cassette had the effect of eliminating S-layer proteins expression altogether, as well as their secretion. Primer extension analysis indicated approximately wild-type levels of *sapA* mRNA in the *sapD* mutant  
35 97-205, indicating that the effect of the *sapD* mutation on the inability to detect SLPs in the cytoplasm was not due to a regulatory effect on S-layer protein gene transcription.



### EXAMPLE 17

#### Secretion of SapA from *E. coli* expressing sapCDEF

To determine whether *sapCDEF* permits the secretion of SapA, the ability of *E. coli* strains carrying pIR100 to mediate the specific secretion of *C. fetus* SapA was tested. *E. coli* C600 cells were isolated that had been transformed with pBGYC1, a pACYC184-derived plasmid containing *C. fetus sapA*, and either pIR100 (*sapCDEF*) or pAMP1 alone (vector control). SapA secretion was assayed using immunoblots to indicate the appearance of SapA in filtered, trichloroacetic acid (TCA)-precipitated culture supernatants prepared from these strains. As expected, both *E. coli* strains produced SapA (Figure 6). Secretion of SapA was detected only in supernatants from *E. coli* cells expressing *sapCDEF* (Figure 6). These results indicate that the *C. fetus sapCDEF* genes were sufficient to allow *E. coli* to secrete SapA.

### EXAMPLE 18

#### Conserved features of S-layer protein C-termini

Proteins that are transported by type I secretion systems do not have the N-terminal signal peptides that are conserved in proteins exported by type II systems. Type I secretory proteins rely upon signals that are located at their extreme C-termini. However, these signals tend to have little primary sequence homology, and their exact structures have been difficult to elucidate. In an attempt to define candidate sequences/structures for C-terminal secretion signals in bacterial SLPs, the C-terminal 70 amino acids of four *C. fetus* SLPs for which the sequences are known (SapA, SapA1, SapA2, and SapB) were aligned.

Several conserved peptides were evident. The sequence GDGS(T/G) was present in three of the four S-layer proteins, with SapA2 possessing a slightly different version (skGST). Similarly, the peptide GxTYVV(V/I)D was present in three of the four SLPs, with the corresponding sequence (GxTYVVda) of SapA2 again being slightly more divergent. Several other conserved amino acid residues also were apparent. The DVIV motifs implicated in protease secretion were not

present per se at the extreme C-terminus of any of the S-layer proteins, although similar sequences (DGSVI) were found at the C-termini of the SapA and SapB proteins. Similar to type I-secreted toxins and related exoproteins, the S-layer proteins had 1-4  
5 hydroxylated residues (S or T) within the C-terminal ten amino acids.

To investigate whether the C-terminal domains of these S-layer proteins also could form similar secondary structures, each of these peptides was analyzed using the  
10 programs of Garnier et al. The predicted C-terminal secondary structures ( $\alpha$ -helix,  $\beta$ -sheet, amphipathic peptides, and turn-forming residues) were then superimposed on the alignment of the 4 C-terminal peptides. Three of these peptides (SapA, SapA1, and SapB) consisted of segments predicted to form sheet-sheet-  
15 helix-sheet, with each of these domains separated by turn-forming. The C-terminal domain of SapA2 was predicted to form a structure of helix-sheet-helix-sheet. Furthermore, the sheet-forming regions of all 4 proteins, as well as the most N-terminal helix-forming region of SapA2, were predicted to be amphipathic.  
20 Taken together, these results suggest that the C-termini of four *C. fetus* S-layer proteins contain conserved sequences and secondary structures that are candidates for secretion signals.

S-layer proteins expression in *C. fetus* is based on the single *sapA* promoter present on an invertible 6.2 kb element,  
25 that is bracketed by inverted repeats and oppositely oriented cassettes, *sapA* and *sapA2*, that are subject to substitution (9). By creation of appropriate mutants and by using selection for phenotypic properties, the present invention demonstrates that DNA rearrangement can involve inversion of this element in  
30 concert with one or more of the tandemly arrayed S-layer protein gene cassettes.

DNA inversion has been believed to involve mutually exclusive promoter or structural gene inversion. Either the promoter inverts relative to fixed structural genes (26,27), or  
35 structural genes invert downstream of a fixed promoter (28-30) permitting expression of two alternate gene copies (10,11). The system of DNA inversion in *C. fetus* is novel because it combines the features of each mechanism as both the promoter and the

structural genes are mobile, which permits the shuffling of complete genes and their ultimate expression (Figure 3). Rearrangement of the S-layer protein gene cassettes permits the organism to vary S-layer protein expression and surface antigenicity allowing for evasion of host immune responses. This inversion system differs from the *Mycoplasma pulmonis* *vsa* gene inversion, which rearranges only incomplete coding regions and demonstrates less sequence stability.

The present invention further indicates that inversion occurs randomly between open reading frames of opposite orientation independent of the size of the intervening DNA segment. The economy of a simple inversion system may be especially useful for *C. fetus* which has a relatively small (1.1 megabase) genome (32); the strict conservation of both coding and non-coding regions related to the *sap* homologs in type A and type B strains (7) is consistent with the importance of this efficient system. The present invention expands the paradigms of DNA rearrangement, in which large gene families of complete open reading frames can reassort by inversion so as to vary the surface protein expression of the microbe.

#### **EXAMPLE 19**

##### **Mutant *C. fetus* strain with cassettes replaced by a heterologous antigen**

Each of the *sapA* homologs is altered so that the central portion of the homolog-specific region is replaced by a DNA cassette encoding a heterologous antigen. Representative examples of cassettes that can be inserted are immunogens related to *Salmonella*, *Campylobacter jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) as well as venereal, gastrointestinal, or respiratory pathogens of humans, cattle, sheep, poultry, horses, swine, and reptiles. The replacement is constructed so that the encoded S-layer protein is a tripartite chimera consisting of the N-terminal LPS-binding domain of the S-layer protein, a central region composed of the heterologous antigen, and a C-terminal segment containing the S-layer protein secretion signal. This protein would be able to be secreted from the cell by means of the C-terminal

secretion signal and into the LPS on the *C. fetus* cell surface, thereby exposing the heterologous antigen to the host immune system. Because the natural cassettes are replaced by the chimerae, one embodiment of the invention is the expectation that  
5 carriage of this organism is substantially shorter than for the wild type strain and thus, self-limiting. This strain should colonize for less than 3 months allowing the host to develop a mucosal and systemic immune response to each of the immunogens.

In another embodiment, one may use a mutant *C.*  
10 *fetus* strain in which all but one of the cassettes is replaced by a heterologous antigen. This embodiment is similar to Example 12 except that one of the cassettes (e.g., *sapA2*) remains in its native configuration but that the others are mutagenized to form chimerae. The advantage of this construction is that one can  
15 induce immunity to *C. fetus* based on the single full-length S-Layer protein produced.

#### EXAMPLE 20

##### Mutant *C. fetus* strain in which *recA* is mutagenized

20 In another embodiment of the present invention, one may mutagenize *recA* so that the RecA protein is not produced and therefore, the DNA rearrangements permitting *sapA* antigenic variation can not occur at any detectable frequency. Thus, the *C. fetus* strain can only produce one of the S-Layer proteins encoded  
25 by one *sapA* homolog. This strain should only colonize the host briefly, i.e., until protective immunity has developed to that homolog. Subsequently, the host immune response should eliminate the organism. Thus, this mutant would provide effective immunity against subsequent *C. fetus* infection and is useful for  
30 vaccination of ungulates (including sheep, cattle and horses) in which infectious abortion and/or infertility can occur after the wild-type infection.

#### EXAMPLE 21

35 *C. fetus* strain in which *recA* is mutagenized and the *sapA* homolog that is being expressed is a chimera involving a heterologous peptide

In this embodiment, a *sapA* homolog is mutagenized to allow expression of a chimeric protein including a heterologous antigen. The strain is then passaged in vitro so that the chimeric homolog is in the expression position and then a *recA* mutation is made. This strain now expresses only the chimeric protein which can serve as a means to immunize a host to that antigen. Since the strain can not vary and is not producing a native S-Layer protein, the duration of colonization in the host is brief (days). This attenuation of the *C. fetus* strain allows safe immunization for the selected antigen.

#### EXAMPLE 22

Mixed mutant *C. fetus* strains each including a *sapA* chimera which is also a *recA* mutant

In this embodiment, a series of mutants are constructed in which for each a single *sapA* homolog is mutagenized with the replacement to encode a different chimeric protein, each one representing a different heterologous antigen. Each mutant is also RecA-deficient due to mutation in *recA*. A host is inoculated with a mixture of two or more of these strains to provide immunization to the requisite antigens. Each strain is short-lived in the immunized host.

#### EXAMPLE 23

Mutant *C. fetus* strain that secretes a heterologous antigen

Each of the *sapA* homologs is altered so that the 5' section of the homolog-specific region is replaced by a DNA cassette encoding a heterologous antigen. Examples of the cassettes that can be inserted are immunogens related to *Salmonella*, *Campylobacter jejuni*, *E. coli* O157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) as well as venereal, gastrointestinal, or respiratory pathogens of humans, cattle sheep, poultry, horses, swine, and reptiles. The replacement is constructed so that the encoded S-layer protein is a bipartite chimera consisting a central region composed of the heterologous antigen and a C-terminal segment containing the S-layer protein secretion signal. This protein would be able to be secreted from the cell by means of the C-terminal secretion signal thereby

exposing the heterologous antigen to the host immune system. Because the natural cassettes are replaced by the chimerae, one embodiment of the invention is the expectation that carriage of this organism is substantially shorter than for the wild type strain  
 5 and thus, self-limiting. This strain should colonize for less than 3 months allowing the host to develop a mucosal and systemic immune response to each of the immunogens.

In another embodiment, one may use a mutant *C. fetus* strain in which all but one of the cassettes is replaced by a  
 10 heterologous antigen. This embodiment is similar to Example 12 except that one of the cassettes (e.g., *sapA2*) remains in its native configuration but that the others are mutagenized to form chimerae. The advantage of this construction is that one can induce immunity to *C. fetus* based on the single full-length S-Layer protein produced.  
 15

The following references were cited herein:

1. Borst, P. & Greaves, D. R. (1982) *Science* **235**, 658-667.
2. Fujimoto, et al., (1991) *Infect. Immun.* **59**, 2017-2022.
3. Beveridge, T.J., Koval, S.F. (1993) Advances in paracrystalline  
 20 bacterial surface layers. *Plenum Press, New York*. 1-344.
4. Blaser, et al., (1988) *J. Clin. Invest.* **81**, 1434-1444.
5. Wang, et al., (1993) *J. Bacteriol.* **175**, 4979-4984.
6. Garcia, et al., (1995) *J. Bacteriol.* **177**, 1976-1980.
7. Dworkin, et al., (1995) *J. Biol. Chem.* **270**, 15093- 15101.
- 25 8. Dworkin, et al., (1995) *J. Bacteriol.* **177**, 1734-1741.
9. Dworkin, et al., (1996) *Mol. Microbiol.* **19**, 1241-1253.
10. Glasgow, et al., (1989) in *Bacterial DNA inversion systems*, eds. Berg, et al., (ASM, Washington, D.C.), pp. 637-659.
11. van de Putte, et al., (1992) *Trends in Genetics* **8**, 457-462.
- 30 12. Tummuru, et al., (1992) *J. Bacteriol.* **174**, 5916-5922.
13. Blaser, et al., (1987) *J. Infect. Dis.* **155**, 696-705.
14. Sambrook, et al., (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.).
15. Birnboim, et al., (1979) *Nucleic Acids Res.* **7**, 1513-1523.
- 35 16. Blaser, et al., (1985) *J. Infect. Dis.* **151**, 227-235.
17. Wang, E. & Taylor, D. E. (1990) *Gene* **94**, 23-28.
18. Trieu-Cout, et al., (1985) *EMBO J.* **4**, 3583-3587.
19. Feinberg, et al., (1983) *Anal. Biochem.* **132**, 6-13.

20. Pei, Z. & Blaser, M. J. (1990) *J. Clin. Invest.* **85**, 1036-1043.
21. Winter, et al., (1978) *Infect. Immun.* **22**, 963-971.
22. Blaser, M. J. & Pei, Z. (1993) *J. Infect. Dis.* **167**, 696-706.
23. Blaser, et al., (1990) *J. Biol. Chem.* **265**, 14529-14535.
- 5 24. Tummuru, M. K. R. & Blaser, M. J. (1993) *Proc. Natl. Acad. Sci. USA*. **90**, 7265- 7269.
25. Gellert, M. & Nash, H. (1987) *Nature (London)* **325**, 401-404.
26. Silverman, et al., (1979) *Proc. Natl. Acad. Sci. USA* **76**, 391-395.
- 10 27. Abraham, et al., (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5724-5727.
28. Marrs, et al., (1988) *J. Bacteriol.* **170**, 3032-3039.
29. Iida, et al., (1982) *EMBO J.* **1**, 1445-1453.
30. Giphart-Gassler, et al., (1982) *Nature (London)* **297**, 339-342.
- 15 31. Blaser, et al., (1994) *Mol. Microbiol.* **14**, 453-462.
32. Salama, et al., (1992) *Int. J. System. Bacteriol.* **42**, 446-450.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

## WHAT IS CLAIMED IS:

1. A mutant *C. fetus* strain useful for vaccinating an animal to *Campylobacter fetus*, wherein said strain is mutated to contain a DNA cassette encoding a heterologous protein.
2. The mutant *C. fetus* strain of claim 1, wherein a *sapA* homolog is altered.
3. The mutant *C. fetus* strain of claim 1, wherein said heterologous protein is a S-layer protein.
4. The mutant *C. fetus* strain of claim 1, wherein the encoded S-layer protein represents a chimera between the native S-layer protein and the peptide encoded by the cassette.
5. The mutant *C. fetus* strain of claim 1, wherein said cassette is selected from the group consisting of *Salmonella*, *Shigella*, *Campylobacter jejuni*, *E. coli* 0157:H7, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and animal pathogens.
6. The mutant *C. fetus* strain of claim 1, wherein said cassette contains a 5' binding region and 3' secretion signal region and wherein said protein is inserted between said binding region and said signal region.
7. The mutant *C. fetus* strain of claim 1, wherein said cassette contains a 3' secretion signal but has no binding region.
8. The mutant *C. fetus* strain of claim 1, wherein said protein is selected from the group consisting of an antigen and a therapeutic agent.
9. A method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of the strain of claim 1.



10. A mutant *C. fetus* strain, wherein *recA* is mutated so that no functional RecA protein is produced, the DNA rearrangements permitting *sapA* antigenic variation occur at a very low frequency and wherein said *C. fetus* strain can only  
5 produce one of the S-layer proteins encoded by one *sapA* homolog.

11. The mutant *C. fetus* strain of claim 10, wherein said strain contains a *sapA* homolog expressing a chimeric protein including a heterologous antigen.  
10

12. A mixture of mutant *C. fetus* strains, wherein each strain includes a *sapA* chimera which is also a *recA* mutant, wherein a single *sapA* homolog is mutated to encode a different chimeric protein representing a different heterologous antigen and  
15 each mutant is also RecA-deficient due to mutation in *recA*.

13. A method of immunizing a host to develop mucosal and systemic immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective  
20 dose of the strains of claim 12.

14. A strain of bacteria modified to express *SapCDEF* genes.

15. The strain of claim 14, wherein said bacterium is  
25 *Escherichia coli*.

16. The strain of claim 14, wherein a heterologous protein is expressed as a chimeric protein composed of sequences of heterologous origin, sequences that direct the secretion of said  
30 chimeric protein to the cell surface and sequences that direct the binding of the secreted chimeric protein to the lipopolysaccharides of the bacterial cell surface via the *sapCDEF* directed type 1 secretory system.

17. A method of immunizing a host to generate immune responses to an immunogen, comprising the step of administering to said host a pharmacologically effective dose of the strain of  
35 claim 14.

1/7

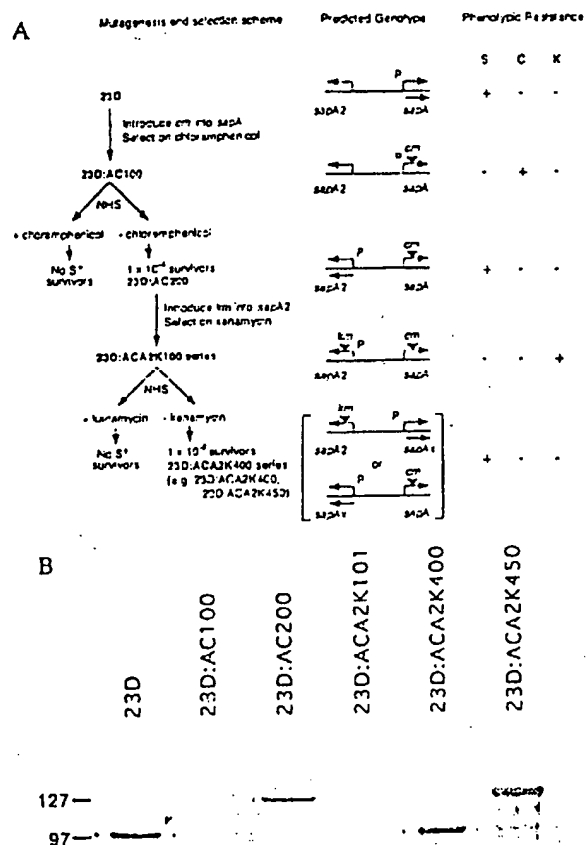


FIGURE 1

**2/7**

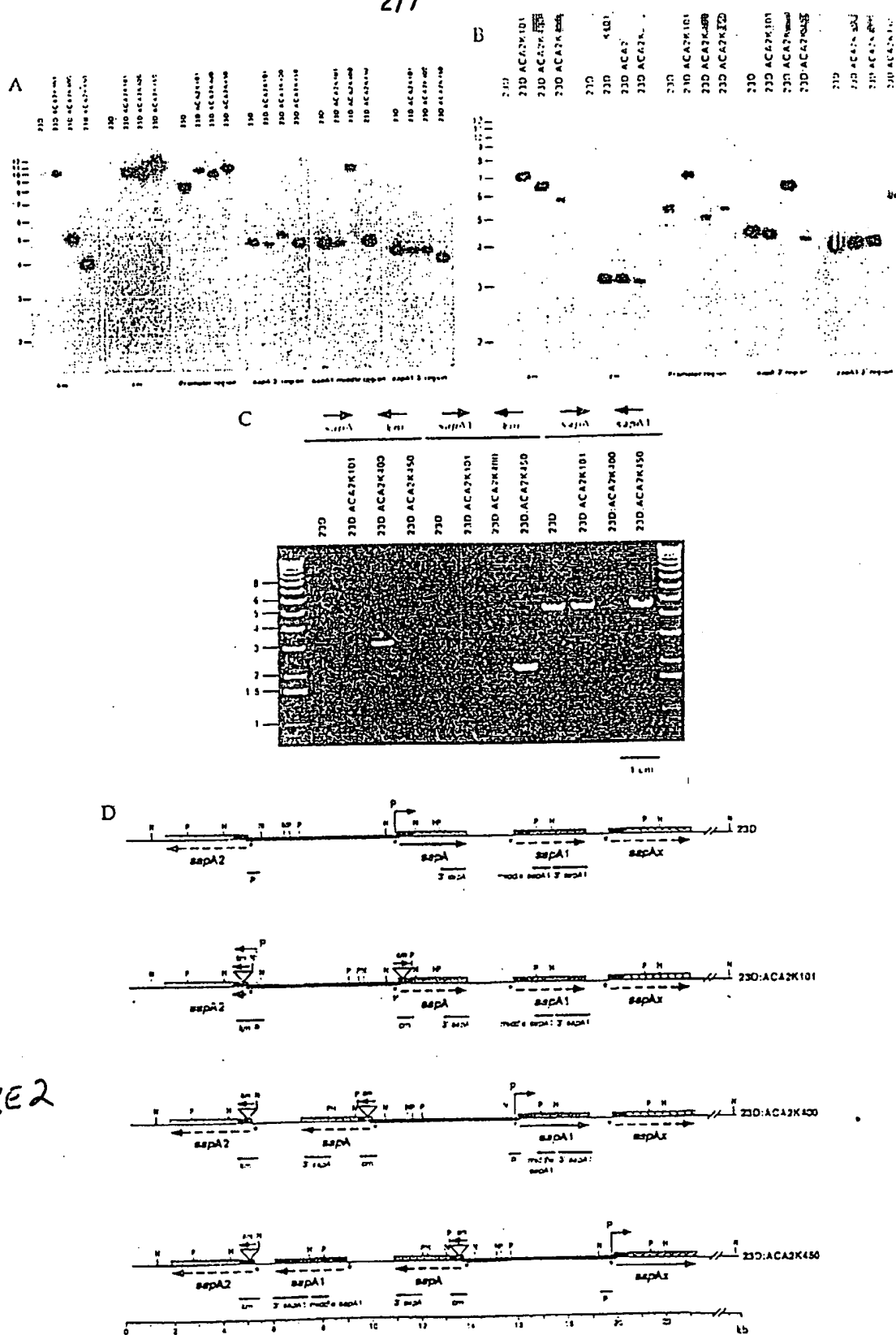


FIG. 2. (Legend appears at the bottom of the opposite page.)

3/7

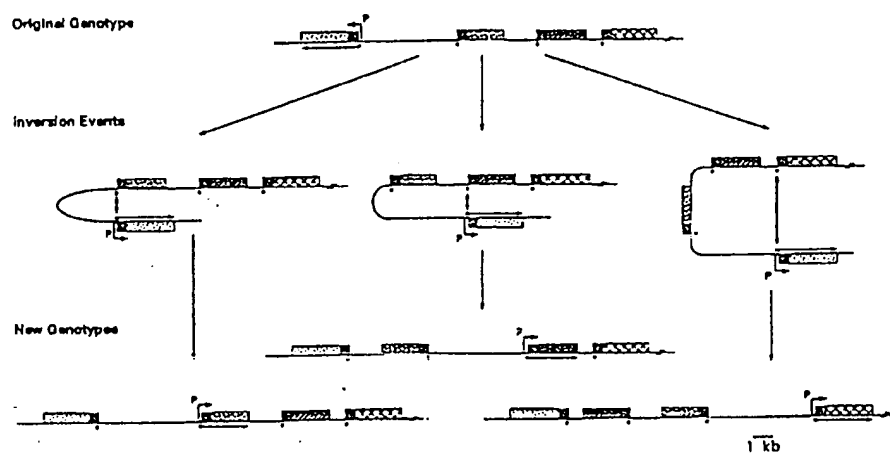


FIGURE 3

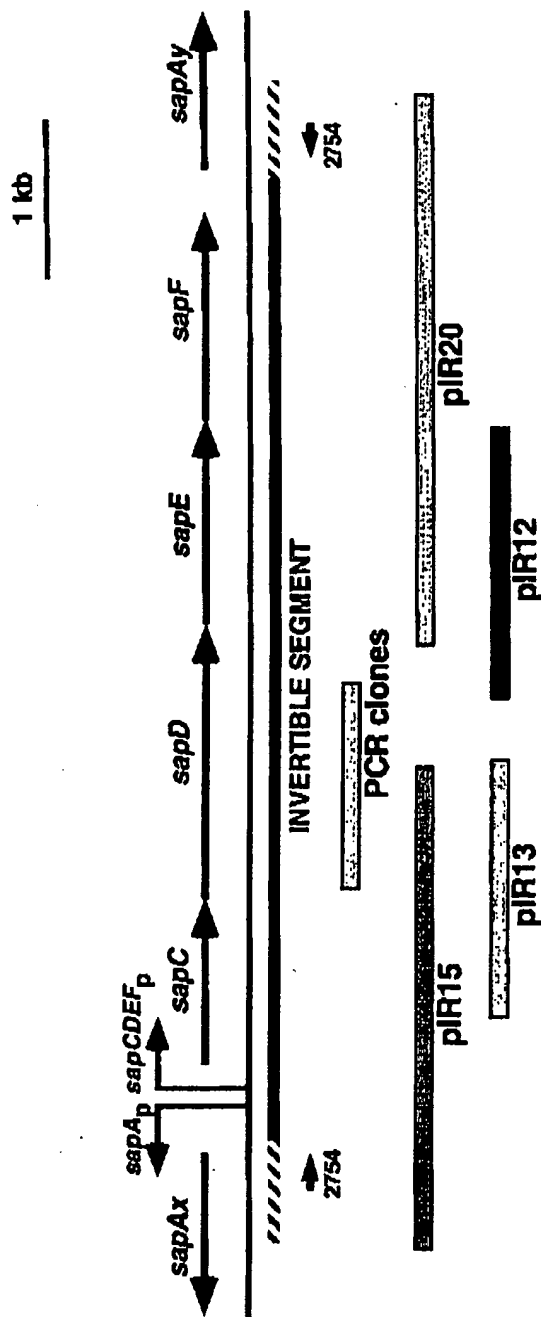


Figure 4

5/7

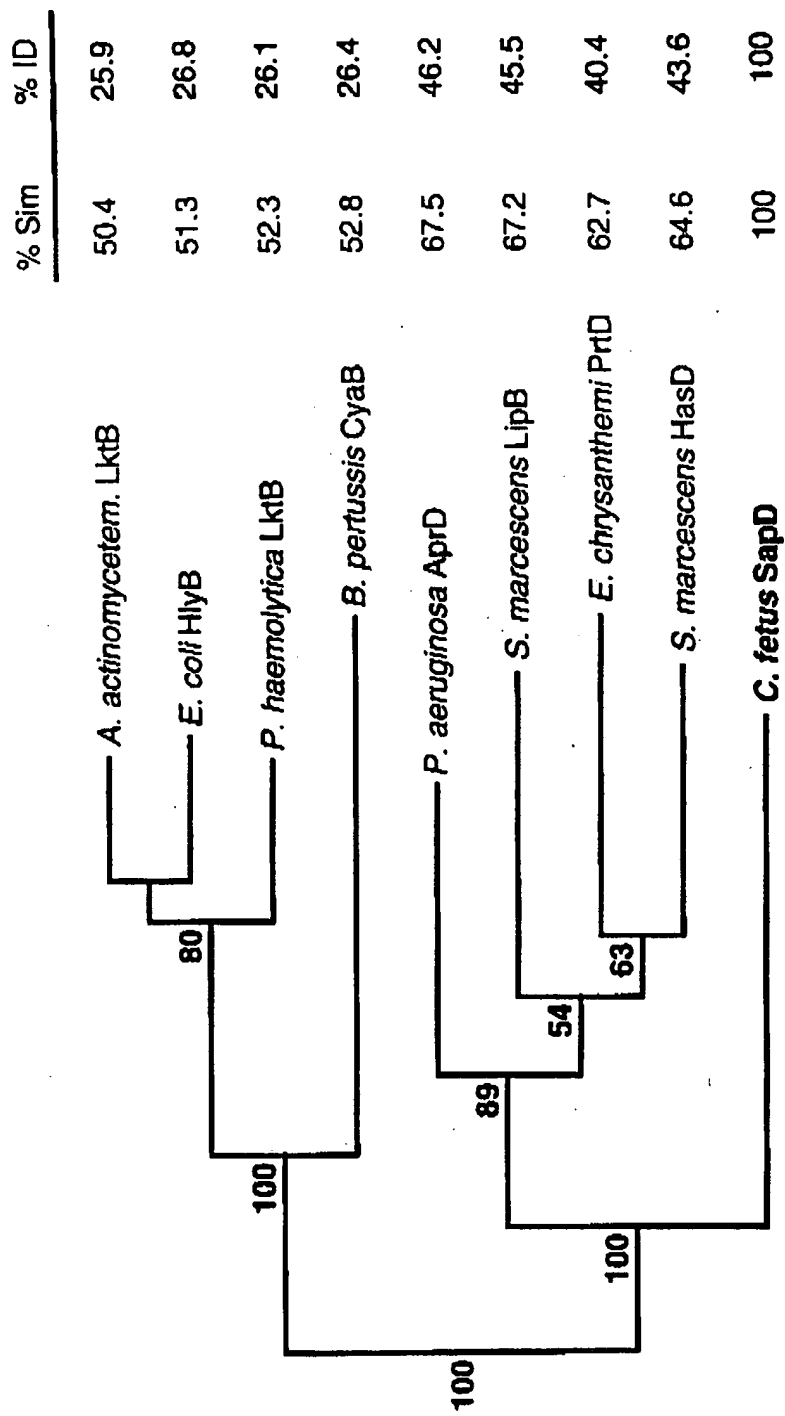


Figure 5

6/7

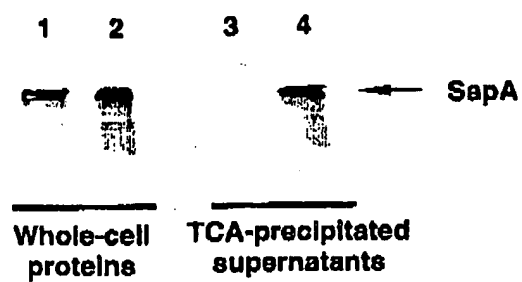


Figure 6

# Use of *C. fetus* as a live vector for antigen delivery to the mucosal immune system

WO 98/33386

PCT/US98/01780

7/7

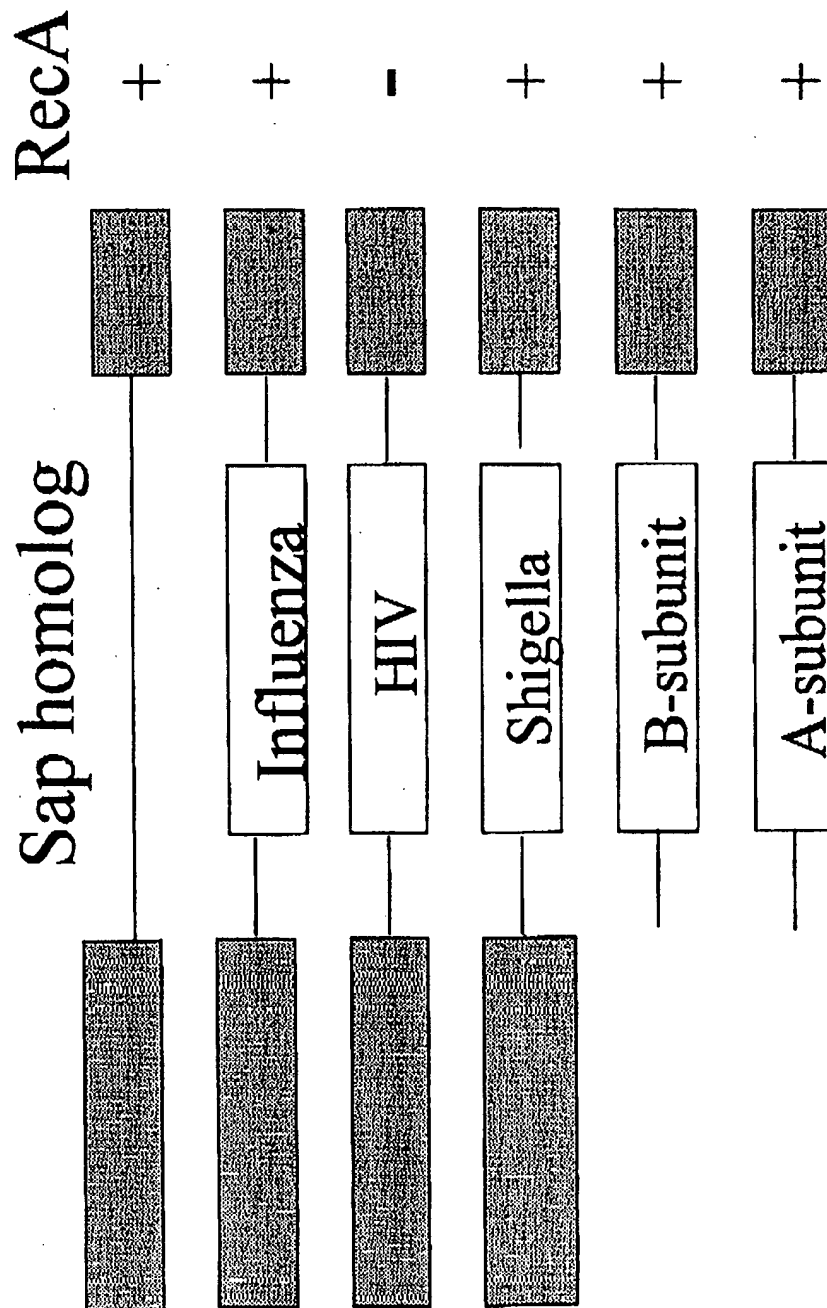


Figure 7



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/01780

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01N 63/00; A61K 48/00; C07H 21/02, 21/04  
US CL : 424/93.2; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS, DIALOG

search terms: sapA, sap(w)a, campylobacter fetus?, mutant?, heterologous, foreign protein?

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DWORKIN, J. et al. Generation of Campylobacter fetus S-layer protein diversity utilizes a single promoter on an invertible DNA segment. Molecular Microbiology, 1996, Vol. 19 (6), pages 1241-53, see abstract.	1-9
Y,P	FUJITA, MASAKI et al. A deletion in the sapA homolog cluster is responsible for the loss of the S-layer in Campylobacter fetus strain TK. Archive of Microbiology, 1997, Vol. 167, No. 4, pages 196-201, see abstract.	1-9
Y	DWORKIN, J. et al. Segmental conservation of sapA sequences in type B Campylobacter fetus cells. Journal of Biological Chemistry. 1995, Vol. 270, No. 25, pages 15093-15101, see abstract.	1-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

31 MARCH 1998

Date of mailing of the international search report

28 MAY 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

GINNY PORTNER

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/01780

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DWORKIN, J. et al. A lipopolysaccharide-binding Domain of the Campylobacter Fetus S-layer protein resides within the conserved N-terminal of a family of silent and divergent homologs. Journal of Bacteriology, April 1995, Vol. 177, No. 7, pages 1734-1741, see abstract.	1-9
Y	FUJITA, M. et al. Localization of the sapa gene on a physical map of Campylobacter fetus chromosomal DNA. Archives of Microbiology, 1994, Vol. 62, No. 6, pages 375-380, see abstract.	1-9
Y	TUMMURU, M.K.R. et al. Rearrangement of sapA homologs with conserved and variable regions in Campylobacter fetus. Proceedings of the National Academy of Science, U.S.A., August 1993, Vol. 90, No. 15, pages 7265-7269, see abstract.	1-9
A	TUMMURU, M.K.R. et al. Characterization of the Campylobacter fetus sapA promoter: evidence that the sapA promoter is deleted in spontaneous mutant strains. Journal of Bacteriology, September 1992, Vol. 174, No. 18, pages 5916-5922, see abstract.	1-9
Y	YANG, LY et al. Reattachment of surface array proteins to campylobacter fetus cells. Journal of Bacteriology, February 1992, Vol. 174, No. 4, pages 1258-1267, see entire document.	1-9
X	BLASER, M.J. et al. High-frequency S-layer protein variation in Campylobacter fetus revealed by sapA mutagenesis. Molecular Microbiology, 1994, Vol. 14, No. 3, pages 453-462, see figure 1 and whole reference.	1-4, 6-9

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/01780

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-9, drawn to a mutant *Campylobacter fetus* strain and a DNA cassette encoding a heterologous protein, wherein the *C. fetus* strain is a *sapA* homolog.

Group II, claim(s) 10-13, drawn to *Campylobacter fetus* mutant strains wherein a *recA* mutation is present together with a heterologous antigen or a *sapA* gene mutation.

Group III, claim(s) 14-17, drawn to any strain of bacteria which has been modified to express SapCDEF genes.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Each group contains mutant strains which evidence specific structural differences in the genes that are altered or added to the mutant strain. Therefore, each group represents mutant strains which have differing special technical features which have differing structures, functions and effects on the over all end product.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**